Evaluation of spermatozoal RNA extraction methods.

Master of Technology Thesis submitted by

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Under the Guidance of

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Supervisor's certificate

This is to certify that the project entitled "**Evaluation of spermatozoal RNA extraction methods.**" submitted by **Sovan Das (214Bm2024)** as master of technology thesis towards the partial requirement for the degree of **Master of Technology in Biotechnology** from the Department of Biotechnology and Medical Engineering at The National Institute of Technology, Rourkela is an authentic work carried out by him under my supervision and guidance. The matter embodied in this report has not been submitted to any other University or Institute for the award of any degree or diploma.

Rourkela,

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Sovan Das

(**214BM2024)**

Declaration of Originality

I, Sovan Das, Roll Number: 214BM2024, hearby declare that this dissertation entitled "Evaluation of spermatozoal RNA extraction methods" present my original work carried out as a M.Tech student of National Institute of Technology Rourkela, and to the best of my knowledge contains no material previously published or written by another person, nor any material presented by me for the award of any degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the section "Reference". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present dissertation.

May 30, 2016
NIT Rourkela

Abstract

The study was aimed to compare different RNA isolation methods from sperm and testicular tissue. A suitable sperm purification technique is required as sperm RNA yield is strongly correlated with the number of sperms. Goat (*Capra hircus*) semen was purified by swim-up method. Motile sperm were selected for the process of RNA isolation. Four different RNA isolation methods namely cold Trizol, Gunidinium thiocyanate method and SDS lysis method and silica column based spin column method were used to isolate total RNA from sperm and testicular tissue. The hot Trizol method yielded good amount of RNA with less contamination of DNA and proteins. The modification of heating the sample in Trizol and an additional chloroform phase separation step increased the yield of total RNA. The modified method yielded highest amount of RNA for both tissue and sperm samples which was 139.5 ng/µL and 38.6 ng/µL respectively. The RNA integrity was high as was defined by a sharp 28S and 18S rRNA band in the agarose gel. The A260/A280 ratio was 2.1 and 1.923 for tissue and sperm samples which signifies purity of RNA. The mRNA was also reverse transcribed and GAPDH amplified. A modified Trizol method was developed which resulted in increased yield (38.60 ng/µL) and purity (A260/A280: 1.923).

Keywords: sperm purification, RNA isolation, A260/A280 ratio, Hot Trizol method, Primer designing, cDNA amplification.

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Chapter 1

Introduction

The function of sperm is to deliver a haploid genome to the oocyte. Sperm cells cannot undergo transcription, yet they contain RNA (Miller *et. al.* 1994). These RNA are mainly fragments of longer transcripts which includes rRNAs and mRNAs (Johnson *et. al.* 2011). Idiopathic infertility is also known as unexplained fertility as the causes of fertility is not known certainly. Previously male infertility was diagnosed by viewing sperm motility, concentration and sperm's morphology. Researchers now have traced the cause to be linked with RNA. Men whose sperm lack critical sperm RNA elements (SRE) have lower chances of naturally conceiving a child (jodar *et al.* 2015). According to this study fertile men carried 648 SRE while infertile men lacked some of them. The more the number of lacking SRE the more he is likely not to conceive a child. Therefore the study of RNA in sperm is a major step in detecting male infertility. Sperm contains very low amount of RNA which is about 0.015 pg/RNA per sample. Each Sperm contains about 4000 mRNA. Further the amount of sperm RNA is overestimated and misinterpreted from contaminating RNAs from other cells present in seminal plasma like leucocytes, somatic cells and cell debris etc. The genomic DNA can also contaminate the result. To overcome these problems different method were adopted for RNA isolation like Guanidium-phenol-chloroform extraction, Trizol method, Hot SDS/Phenol method, hot acid phenol method. Different spin column based method are commercially available in the form of kits.

In order to extract sperm RNA, the sperm purification method must be properly done, so that other somatic cells from seminal plasma does not hamper the result. There are several types of sperm purification methods. Swim-up, swim- down, double swim-up and density gradient centrifugation are the most common methods. Electrophoresis and fluorescence cell sorting methods, Magnetic activated cell sorting can also be used for sperm isolation from semen. The extraction methods for RNA can be generally classified into organic extraction methods, spin basket methods, magnetic particle based methods and direct cell lysis methods. The guanidinium-isothiocyanate-phenol-chloroform extraction is a direct liquid-liquid extraction

method. (P. Chomczynski and N. Sacchi, 1987). A mixture of phenol-chloroform-isoamyl alcohol for extraction of RNA is manufactured commercially, by the brand name Trizol.

Trizol separates RNA to the upper aqueous phase and phenol being of higher density forms the lower organic phase. Phenol denatures the proteins and guanidium isothiocyanate being a chaotropic agent also denatures proteins. Proteins have hydrophobic cores and are hydrophilic outside, but in the presence of phenol the hydrophobic core comes outside and the protein is denatured. In neutral pH, both DNA and RNA separates into the aqueous phase, but in acidic pH DNA becomes less soluble in water and separates into the interphase.

Efficient removal of sperm from semen has to be performed for expecting a higher and appropriate RNA yield. Separation of spermatozoa from seminal plasma is very important for estimating RNA from sperm cells and to prevent contamination from other somatic cells, leucocytes and cell debris. Methods for purifying sperm cells from semen include swim-up, double swim-up, swim down and Density gradient centrifugation.

Isolating RNA is much difficult than isolating DNA as RNA is short lived. The ubiquitous presence of RNases makes isolation a much difficult process. Autoclaving of micropipette tips and centrifuge tubes cannot itself inactivate RNases, as the RNases renatures as the solution cools, however RNase inhibitor Diethyl pyrocarbonate (DEPC) treatment inactivates RNases. Obtaining high quality RNA is important as this is used in further downstream processes like RT-PCR, RLM-RACE, cDNA synthesis, microarray analysis and RNA sequencing.

Presence of RNA in sperm nucleus was first shown by pessot *et al* in 1988. They labelled the nucleus with RNase-colloidal gold, which only stained only the nucleus region but the tail part of the sperm was not stained. Sperm plasma membrane distinguish themselves from somatic cell plasma membrane by the presence of di-sulphide bonds which crosslinks the proteins. The usage of DTT (dithiothreitol) can effectively lyase the sperm plasma membrane.

Chapter 2

Review of Literature

When spermatids differentiate into spermatozoa there is a change in chromatin structure due to DNA compaction. This is caused as histones are replaced by protamines, followed by loss of cytoplasm. Before meiotic division there is a high level of transcriptional activity and transcriptional activity decreases after meiosis at the stage of round spermatid (Dadoune *et. al.* 2004). The translational activity in spermatozoa is also compromised as it has been shown spermatozoa harbour no or few ribosomal RNAs (Ostermeier et al. 2002, Grunewald *et al*. 2005). It has been proposed that due to time delay of transcription and translation, during spermatogenesis, the mRNA population in spermatozoa should be representative of the past events of spermatogenesis (Ostermeier *et al.* 2002, Dadoune *et. al.* 2004, Steger 2003, Lambard *et al.* 2004). mRNA is generally present in sperm's mid-piece region, tail region (Kumar *et al.* 1993) as well as within nuclear compartments of the spermatozoa (Pessot *et al.* 1989, Wykes *et al.* 1997).

The isolation of RNAs has also been subjected to many chemical treatments. Rat liver was processed for RNA isolation by Guanidium chloride with additional phenol separation step but the yield in this case was only 20-30 % of total tissue RNA (Volkin & Carter, 1951). Using this finding, Kirby demonstrated the use of phenol to separate nucleic acids from proteins in 1956. Thereafter guanidinium extractions were the method of choice for RNA purification, replacing phenol extraction. The use of guanidinium thiocyanate instead of guanidinium chloride was first briefly mentioned by (Ullrich *et al.* 1977), and later successfully employed by Chirgwin *et al.* in 1979.

Chirgwin *et al.* used guanidinium thiocyanate to isolate undegraded RNA from ribonuclease-rich tissues like pancreas. A combination of guanidinium thiocyanate and hot phenol for RNA isolation was reported by Feramisco *et al.* in 1981. Then the Single-step method of RNA isolation was developed by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski, P. &Sacchi, N. 1987) which describes about the isolation of RNA using acid guanidinium thiocyanate-phenol-chloroform extraction. It is a liquid-liquid extraction method

which employs a pink colour liquid called TRIZOL™ that highly inactivates the RNases. It uses acidic Phenol-chloroform (5:1) and after centrifugation steps the RNA ends up in upper aqueous phase, DNA in middle interphase and proteins and other cell debris in lower organic pink colour phase. Guanidium Thiocyanate disrupts cells solubilises their components and denatures endogenous RNases simultaneously. Chloroform and phenol denatures proteins. Phenol retains 10-15% of the aqueous phase, so chloroform is used to prevent the retention of phenol by water and improve RNA yield. An acidic pH is required so that DNA separates to interface region after centrifugation. The mixture of phenol chloroform is used to increase the efficiency nucleic acid separation. Chloroform helps to prevent cross contamination of aqueous phase with organic phase. Isoamyl alcohol is used with phenol-chloroform to prevent foaming. Isopropanol helps in precipitation of RNA.

The method gives high yield of RNA and takes less than 4 hrs. Using this method large number of samples can be processed using small amount of cells or tissue. Purification of human sperm by a discontinuous Percoll density gradient with an innercolumn was done by (S kaneko, Soshio, Kkobanawa, Tkobayashi, 1986). Percoll gradient was prepared in a concentration from 30-80% which was then centrifuged at 600 g for 30 mins using a swing out rotor. After centrifugation sperm pellet was taken from the bottom which yields high concentration of motile sperms. As it had an inner column the contamination of sperm by seminal plasma was negligible, estimated by protein, Fructose and alkaline phosphatase activity (M Mahadevan, G Baker, 1984).

A molecular analysis of population of mRNA in spermatozoa was done to examine RNA integrity, mRNA in spermatozoa and spermatids. RNA integrity assessment was done by three methods- micro-electrophoresis, comparative smearing after global amplification and PCR amplification of target sequence located in either 3’ or 5’ ends (Gilbert *et al.* 2007).

Spermatozoal total RNA could be contaminated by RNA from other testicular cells. Testis contains several other cell types, namely, Leydig cell, sertoli cell, myoid cell and different germ cells. Literature was reviewed to select cell specific Markers for different cell types in Testis depicted in the table below.

Table 1: Different cell specific markers for testicular and germ cells.

Cell Type	Markers
Leydig	calretinin, cyp17
Sertoli	sox9, GATA4, stra8
Myoid	αSMA, Myh11, calponin
Primary spermatocyte	Kit, dazl, sycp3
Spermatogonia	Plzf, thy11
Sperm	Promine-1, Protamine-2, clustrin,

In spin column method RNA binds to a silica gel membrane which is present inside a column. It is a solid phase extraction method which uses the property of RNA binding to the column under certain conditions of pH and salt concentration. The pH for extracting RNA is kept acidic. Firstly, the tissue or cells are lyased homogenized with a lysis solution. The lysis solution is mainly made up of chaotropic agents such as Guanidium thiocyanate. The binding column along with ethanol is used as a binding solution and after a centrifugation step, the RNA is washed with ethanol to remove impurities such as proteins and DNA. Lastly an elution step is performed, to elute the RNA from the spin column.

The SDS lysis method uses the solubilizing property of sodium dodecyl sulphate (SDS) and denaturing property of phenol. Besides it dissolves the membrane and weakens the interaction between protein and nucleic acids.Chloroform is used for phase separation. Protenase k is also used as a denaturing solution. This method is generally used to extract RNA from tissue cultured cells.

To check integrity of RNA, it is generally run on an agarose gel with TAE or TBE buffer. RNA fragments are separated on the basis of their molecular weight by applying an electric field. RNA is visualized by staining with ethidium bromide. Xyelene cyanol and bromophenol blue are used as tracking dye in RNA gel loading buffer.

Chapter 3

Objectives

The specific objective of the present study were:

1. To evaluate sperm purification methods.
2. To evaluate sperm RNA by different methods that were comparatively used for somatic cells.
3. To compare different RNA isolation methods for sperm and Tissue on the basic of quantity, purity and integrity which was analysed by (Multi-skan GO Spectrophotometer) and agarose gel electrophoresis.
4. To validate the sperm RNA purity by PCR amplification of housekeeping gene.

Chapter 4

Material and Methods

I. Sample collection and storage

Goat (*Capra hircus*) testis samples were collected from local abattoir within 2 hour of slaughter and transported in saline at 4^{o} C. Upon arrival in the laboratory, testis were washed thoroughly with saline and rinsed briefly with 70% (v/v) Ethanol (Hi-Media, Mumbai, MB105) and kept in fresh saline. Semen was then collected by flushing with warm 35^{o} C PBS (Hi-Media, Mumbai, ML023) using a 20 gauge needle fitted to hypodermic syringe (Fig. 1). 20 µL of cell suspension was used for counting number of sperm cells using a Haemocytometer. The rest sperm sample was centrifuged and the sperm pellet was stored in 50 µL RNAlater™ (Invitrogen, California, 1504002) for further use.

Fig 1. Dissection of testis and extraction of sperm from epididymis. i: Collected testis sample, ii: removal of Tunica albuginea, iii: injection of PBS in epididymis, iv & v: collection of sperm in PBS, vi- collection of testis tissue sample.

II. Estimation of sperm concentration

Estimation of sperm concentration was done using a Haemocytometer. 20 µL of suspension was pipetted into the space below the coverslip and used for counting with an inverted microscope. Cells were counted in sixteen small square in each four corner squares. 10 µL of cell suspension was diluted with 990 µL of fresh PBS.

Total number of cells = (Total number of cells counted x (Dilution Factor/Number of big square) x 10^4) cells/ml.

Sperm Purification method: Swim-up method

Sperm samples were processed in warm 1X PBS by conventional swim-up method, for selecting motile sperms free of somatic and immature spermatocytes. Sperm cell suspension was centrifuged at 2000 g for 10 minutes. An aliquot soft pellet was resuspended at the bottom of another fresh 15 ml centrifuge tube (Tarsons, Kolkata, 546021) containing 1X PBS (Hi-Media, Mumbai, ML023) at 37^{o} C. The tube was incubated at 37^{o} C in a slanting position of 45° angle for 1 hour in a 5 % carbon dioxide incubator (New Brunswik, Hamburg, Eppendorf). The top supernatant was removed and accessed for sperm motility, sperm morphology and sperm viability.

Isolation of spermatozoal RNA

Sperm RNA was isolated by five different methods namely Guanidium Isothiocyanate method, SDS Lysis Method, Cold Trizol Method, Spin Column Method and modified hot Trizol method. All the methods varied on the lysis condition and the number of steps involved to extract RNA from tissue or sperm cells. 250µl of cell suspension containing 2 x10^{8} cells and 80 mg of testicular tissue was used to extract total RNA from sperm cells and testis tissue respectively.

Guanidium Isothiocyanate method

Reagents: The denaturing solution (solution A) was a mixture of 4M Guanidium Thiocyanate (Hi-Media, Mumbai, MB015), 25mM sodium citrate (Hi-Media, Mumbai, MB048), pH 7; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. A stock solution of denaturing solution was prepared as follows: 4.74 gm of Guanidium Thiocyanate (Hi-Media, Mumbai, MB015) was dissolved in 5 ml distilled water mixed with 0.35 ml 0.75 M sodium citrate, pH 7 (Hi-Media, Mumbai, TC249); 0.50 ml 10% sarcocyl (Hi-Media, Mumbai, MB108), at 65^{o}C. Solution B was prepared by adding 72 µl 2-mercaptoethanol (Hi-Media, Mumbai, MB041) per 10 ml of stock solution.

Experimental Procedure: 250µl of cell suspension containing 2 x10^{8} cells was used to extract total RNA. Cells were pelleted by centrifuging at 3000 g for 5 min at 4˚C. 1 ml of solution A was added to the soft sperm pellet and was homogenized at 20˚C for 30 seconds and the

homogenized sample was incubated at 4˚C for 1 hr. The content was later mixed thoroughly by inverting the tubes. Subsequently, 0.1 ml of 2 M sodium acetate (Hi-Media, Mumbai, MB048), pH 4, and 0.2 ml of chloroform: isoamyl alcohol (49:1) (Hi-Media, Mumbai, MB115) were added to the homogenate and vortexed virorously for 10 seconds, followed by incubation on ice for 15 minutes. Subsequently the sample was centrifuged at 10,000 g for 20 minutes at 4˚C. The aqueous phase was later transferred to a fresh 1.5 ml tube and an equal volume of isopropanol (Hi-Media, Mumbai, MB063) was added. The mixture was kept for overnight incubation at -20˚C for precipitation of RNA. On the next day, centrifugation at 10,000 g for 20 minutes at 4˚C was performed. The supernatant was discarded and the pellet was dissolved in 0.3 ml of solution B. An equal volume of isopropanol (Hi-Media, Mumbai, MB063) was added and centrifuged again at 10,000 g for 10 minutes. The pellet was again resuspended in 75 % (v/v) Ethanol (Hi-Media, Mumbai, MB106), sedimented, air dried for 15 minutes and dissolved in 30 µl of nuclease-free-DEPC treated water (Hi-Media, Mumbai, TCL016) was used to dissolve the pellet. The yield of extracted RNA was calculated by measuring the absorbance at 260 nm. An absorbance of 1 at 260 nm was considered to be 40 µg of RNA per ml. The Ratio of the absorbance at 260 nm, 280 and 230 nm was used to evaluate the purity of the RNA sample. A260/A280 ratio of 1.9 - 2.1 is generally considered “pure” RNA. The ratio of A260/A280 and A260/A230 are used to evaluate the protein and phenol contamination of the RNA sample.

SDS Lysis method

Reagents: A mixture of 2 % SDS and 16 mM EDTA in water was used as a lysis solution. A stock of lysis solution was prepared as follows: 1ml of 20% SDS (Hi-Media, Mumbai, ML007) and 320 µl of 0.5 M EDTA (Amresco, Dallas, E117)) was mixed and volume was made up to 10 ml using nuclease-free-DEPC treated water (Hi-Media, Mumbai, TCL016). The lysis solution was heated at 100^{o} C during its use.

Experimental procedure: The sperm pellet was centrifuged and 500 µl of heated chloroform at 65˚C and 500 µl of heated SDS lysis solution was mixed with the sperm pellet and vortexed at high speed to efficiently homogenize the sperm cells. The content was centrifuged at 10,000 g for 20 minutes for phase separation. The aqueous phase was later transferred to a fresh 1.5 ml centrifuge tube and an equal volume of chloroform (Hi-Media, Mumbai, MB109) was added. It was then vortexed at high speed and centrifuged at 10,000 g for 15 minutes at 4˚C. The aqueous

phase was again transferred to a new 1.5 ml tube and an equal volume of chloroform: isoamyl alcohol (24:1) (Hi-Media, Mumbai, MB115) was added. The aqueous phase was transferred to a fresh 1.5 ml tube and an equal volume of ice cold isopropanol (Hi-Media, Mumbai, MB063) was added and incubated at -20˚C in a deep freezer for overnight. Next day the supernatant was discarded and the pellet was washed with 75% (v/v) ice cold ethanol (Hi-Media, Mumbai, MB106) by centrifuging 7500 g for 5 minutes. The pellet was dissolved in 30 µl DEPC treated nuclease free water (Hi-Media, Mumbai, TCL016) and heated on a dry bath at 65˚C to dissolve the pellet. The RNA sample was evaluated for yield and purity, by evaluating absorbance at 260 nm, 230nm and 280 nm. The integrity of RNA was checked by running the RNA in 0.8% denaturing agarose gel.

Cold Trizol method

Reagents: RNA-Xpress™ Reagent (Hi-Media, Mumbai, MB061),

Experimental procedure: Trizol method was performed as per manufacture's protocol with slight modifications. Briefly, 250µl of sperm suspension was taken in a 1.5 ml centrifuge tube and to the soft sperm pellet 1 ml of RNA Xpress Reagent (Hi-Media, Mumbai, MB061) was added. It was homogenized with a micro pestle and incubated at room temperature for 5 minutes. Subsequently, 200µl of chloroform (Hi-Media, Mumbai, MB109) was added and mixed vigorously by hand for 15 seconds and cooled on ice for 15 minutes at room temperature. Sequentially phase separation were obtained by centrifuging the contents at 10,000 g for 15 minutes at 4˚C. The colourless upper aqueous phase was pipetted and transferred to a new 1.5 ml RNAse-free centrifuge tube (Tarsons, Kolkata, 500016), followed RNA precipitation by adding 500 µl of ice cold isopropanol (Hi-Media, Mumbai, MB063) to the aqueous phase, mixed vigorously by hand to mix the contents and incubated overnight at -20˚C in a deep freezer. Next day, the supernatant was discarded by upside down of the tube. RNA pellet was ethanol washed by adding 75% (v/v) ethanol (Hi-Media, Mumbai, MB106) and centrifuged at 7500 g for 5 min at 4˚C. The supernatant was discarded and the pellet was kept to air dry at room temperature. The pellet was dissolved in 30µl nuclease free DEPC treated water and heated at 65˚C for completely dissolving the pellet. The yield and purity of RNA was estimated by taking absorbance reading at 260nm, 280 nm and 230nm in a spectrophotometer (Multiskan GO,

Thermo Fisher scientific, Masssachusetts, 51119200) by pipetting 2µl of the sample in a micro plate reader with nuclease-free-DEPC treated water (Hi-Media, Mumbai, TCL016) as blank.

Spin Column method

Reagents: The kit contains-

Product code	Reagents provided
DS0037	RNA lysis solution (HRL)
DS0041	Prewash solution (RW1)
DS0012	Wash solution concentrate (WS)
DS0042	Elution solution (Rnase-free water)
DBCA03	HiElute Miniprep spin column capped
DSCA02	HiShredder
DBCA016	Collection tube uncapped, polypropylene 2 ml
PW1139	Collection tube, polypropylene (2 ml)

10µl of 2-mercaptoethanol to 1 ml of Lysis solution (HRL) was added. Wash solution II was diluted by adding 7 ml 100% Ethanol to 1ml WS solution.

Experimental procedure: HiPura™ Total RNA Miniprep Purification Kit (Hi-Media, Mumbai, MB602) was used to extract spermatozoal RNA as per manufacture's protocol with slight modifications. 250µl cell suspension was taken in a 1.5 ml centrifuge tube and sperm cells were pelleted by centrifuging for 5 min at 3000 g. Cell pellet was loosened by flicking the tube and 600 µl RNA lysis solution (HRL) was added directly to the cell pellet. The lysate was directly pipetted into a HiShredder (DSCA02) placed in a 2 ml uncapped collection tube, and centrifuged for 2 minutes at 10,000 g. An equal volume of 70% ethanol was added to the homogenized lysate collected in uncapped tube after centrifugation, and mixed well by pipetting. Lysate was loaded

into HiElute Miniprep spin column, centrifuged for 30 seconds at 10,000 g. Flow through was discarded and 700 µl of Prewash Solution (RW1) was added to the HiElute Miniprep Spin Column and centrifuged at 10,000 g for 1 minute. Flow-through was discarded. The collection tube was taken and 500 µl of Wash Solution was used to wash the column by centrifuging it for 1 minute, 10,000 g at 4^{o} C. A second washing step was done with 500 µl of Wash Solution followed by centrifugation for 2 minutes at 10,000 g to dry the membrane. The HiElute Miniprep Spin column was transferred to a new 2 ml capped collection tube and 30 µl Elution Solution was added directly onto the HiElute Miniprep Spin column. The tube was closed gently, and centrifuged for 1 minute at 13,000 rpm to elute the RNA. Absorbance reading at 260nm and 280 nm was measured, which were used to measure the yield and purity of the isolated RNA.

Modified Hot Trizol method

Sperm pellet was recovered by centrifuging 250 µl of sperm suspension and 1 ml of RNA Xpress Reagent was added directly to it. The tube was heated at 65˚C for 15 minutes with repeated vortexing to lyase the sperm cell membrane. Cells were homogenized with a micro pestle and incubated at room temperature for 5 minutes. Subsequently 250µl of chloroform (Hi-Media, Mumbai, MB109) was added and mixed vigorously by hand for 15 seconds, followed by incubation on ice for 15 minutes at room temperature. A centrifugation step at 10,000 g for 15 minutes at 4˚C was performed to separate the phases. The upper aqueous phase was pipetted and transferred to a fresh 1.5ml RNAse-free centrifuge tube and an equal volume of chloroform: isoamyl (24: 1) alcohol (Hi-Media, Mumbai, MB115) was added which was followed by an incubation of 10 minutes at room temperature. It was then centrifuged at 10,000 g for 10 minutes at 4˚C. In a similar way the upper aqueous phase was transferred to a fresh tube and 500 µl of ice cold isopropanol (Hi-Media, Mumbai, MB063) was added to it, which was mixed vigorously by inverting the tube. The tube was incubated overnight at -20˚C in a deep freezer. Next day, it was centrifuged at 10,000 g for 10 minutes at 4˚C. The supernatant was discarded by upside down of the tube, the pellet was ethanol washed with 70% (v/v) ethanol (Hi-Media, Mumbai, MB106) by centrifuging at 7500g for 5 min at 4˚C. The supernatant was discarded and the pellet was kept to air dry at room temperature. The pellet was dissolved in 30 µl nuclease-free-DEPC treated water (Hi-Media, Mumbai, TCL016) and heated at 65˚C to completely dissolve the pellet.

Isolation of RNA from testicular tissue

Similar methods were followed for RNA isolation from tissue, as done for sperm RNA. 50mg of testis tissue were taken for isolation of tissue RNA whereas 250µl of Sperm has been used for spermatozoal RNA isolation. For tissue sample precipitation time was also differen,t as overnight incubation was avoided in case of tissue sample and incubation was done for 30 minutes at -20˚C. Then centrifugation and elution steps were followed same as sperm RNA isolation methods.

First strand cDNA synthesis and PCR

Total RNA was subjected to reverse transcription using High Capacity cDNA Reverse Transcription Kit (Invitrogen™, Carlsbad, California, 4368814) as per manufacture's protocol with slight modification. cDNA template was prepared by reverse transcriptase-PCR using the kit and 10 µl of total RNA. 2X RT reaction master mix was prepared in a 0.2 ml PCR tube (Hi-Media, Mumbai, CG283) by adding the following ingredients.

Table 2: Preparation of cDNA 2X master mix.

Component	1 Volume (µL)
10X Buffer	2
25X dNTP (100 mM)	0.8
10 X Random Primer	2`
DEPC treated Nuclease free water	4.2
MultiScribe™ Reverse Transcriptase	1
Total	10

Polymerase chain reaction were performed in a Thermal cycler (Veriti, Applied biosystems), with denaturing, annealing and extension temperature of 25º C, 37 º C and 85 º C for 10 minutes, 120 minutes and 5 minutes respectively.

To verify the isolation of mRNA, amplification of the housekeeping gene GAPDH was performed. The Reaction master mix was prepared in a tube which contained 1.5 µl of forward and reverse GAPDH primer, 1 µl of cDNA, 12.5 µl AmpliTaq Gold 360™ (Invitrogen, California, 4398881) and 8.5 µl DEPC-treated-nuclease-free-water. (Invitrogen, California, 4398881). The sequence of forward and reverse GAPDH primer was GAGTCAACGGATTTGGTCGT and TGTGGTCATGAGTCCTTCCA respectively. The reaction mixture was subjected to an initial denaturation of 7 minutes at 95º C, this was followed by 30 cycles of polymerase chain reaction. Each cycle consisted of a denaturing, annealing and extension step at 95 º C, 57 º C, 72 º C for 30 seconds, 30 seconds and 1 minutes respectively. This was followed by a final extension for 7 minutes at 72 º C.

Verification of sperm purity

To verify total RNA was free of RNA from contaminating cell types, primers for different cells were designed. Sperm specific markers include Promine-1, Protamine-2, clustrin, calmegin etc. Primer for sperm specific marker were made along with markers of Leydig cells, sertoli cells, Myoid cell and primary spermatocyte. Primer designing was done using NCBI primer designing tool and the primers was further analysed using Netprimer tool (Biosoft Net) for its melting Temperature and its secondary structure which includes hairpins, self-dimer and cross dimer.

Chapter 5

Result

Yield of sperm and its purity

Number of sperm cells was found to be 7 x 10^8 per ml of suspension. For extraction of RNA 250 µl of cell suspension (2 x 10^8cells) were used for all the methods. Similar procedures were followed to isolate RNA from 50mg of Testicular Tissue.

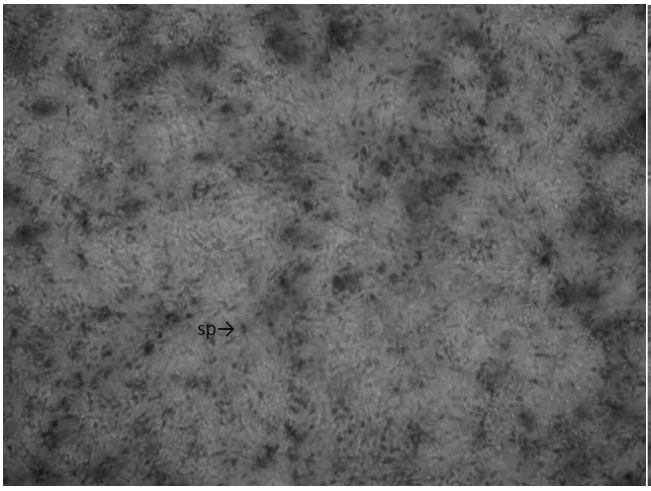

Fig 2. Sperm cells in suspension before swim-up; (sp: Sperm cells)

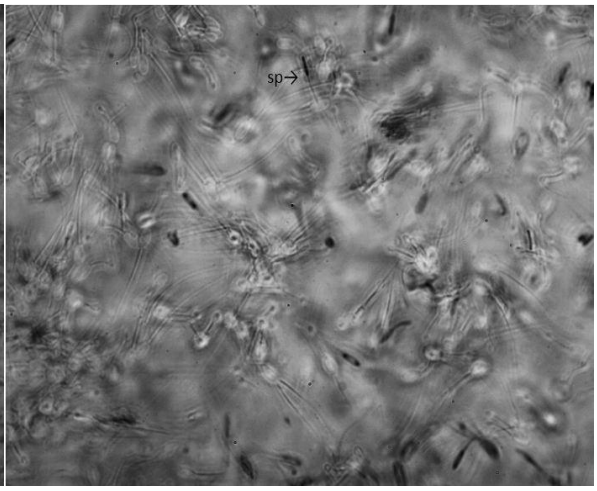

Fig.3. Sperm cells after swim-up (only motile sperms present) (sp: Sperm cells)

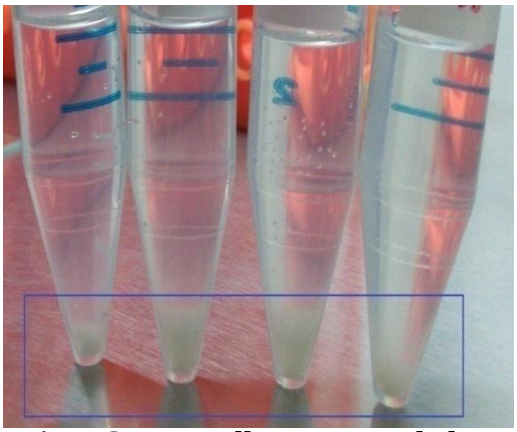

Fig 4. Sperm pellet resuspended at the bottom of 15 ml tubes for swim-

Sperm pellet collected after dissections were purified by swim up method to observe the motile sperms which was represented by fig 3. Sperm count decreased after purification, as only few motile sperms were able to travel to the top layer through 1X PBS.

RNA isolation from sperm sample

Aqueous phase separated by different methods were observed in figure 5-8. Formation of protein complex layer was more prominent in case of SDS lysis and GITC method, whereas spin column method produced less amount of protein complex formation. Aqueous phase formed in case of Trizol method and Spin column method was found to be less as compared to SDS and GITC method.

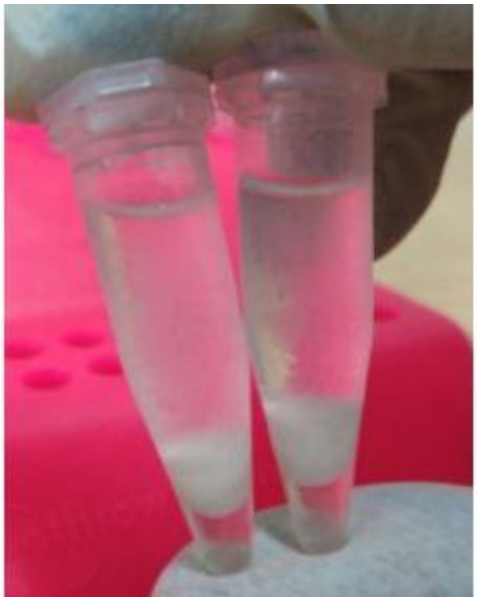

Fig. 5. Phase separation in GITC method for sperm sample

Fig. 6. Phase separation in SDS lysis method for sperm sample

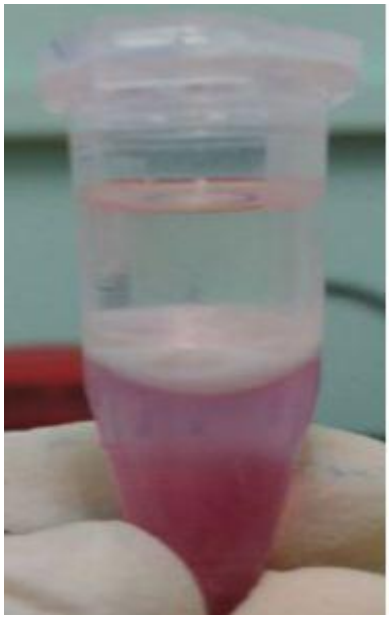

Fig. 7. Phase separation in cold Trizol method for sperm sample

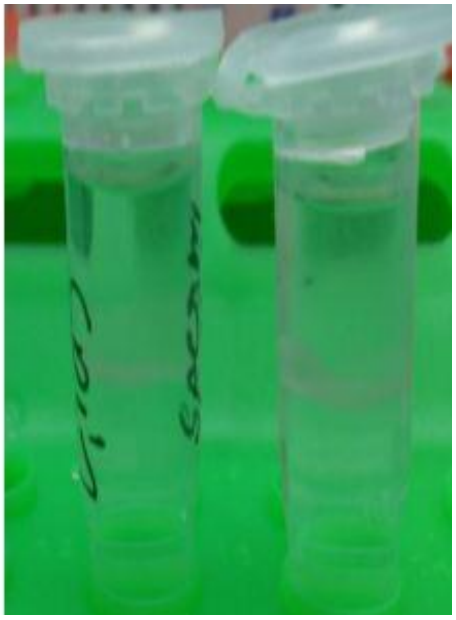

Fig. 8. Phase separation in spin column method for sperm sample

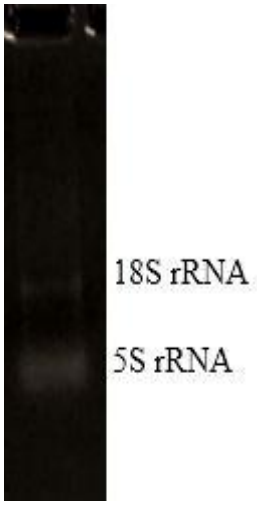

Fig. 9. RNA from testis tissue by GITC

Fig. 10. RNA from sperm sample by GITC

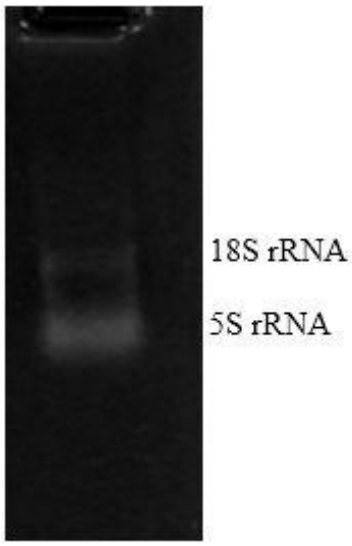

Fig. 11. RNA from testis tissue by SDS

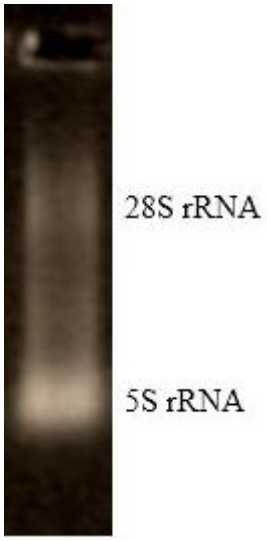

Fig. 12. RNA sperm sample by SDS

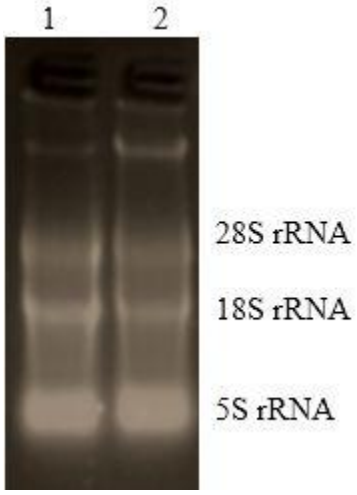

Fig. 13. RNA from testis tissue by 1. cold RNA Xpress reagent and 2. Hot RNA Xpress reagent method.

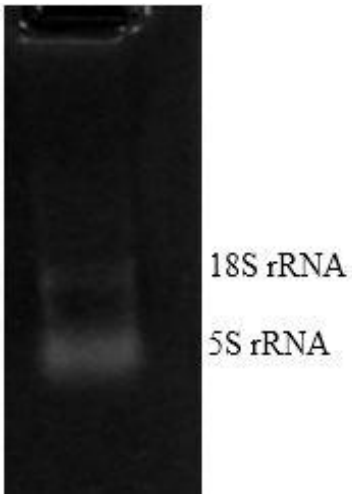

Fig. 14. RNA from sperm sample by cold trizol

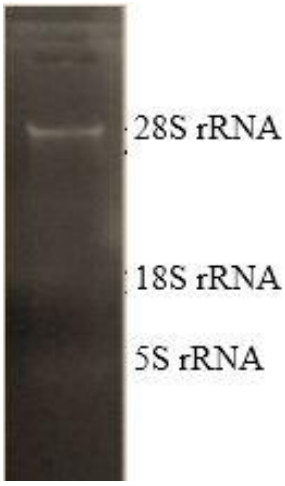

Fig.15. RNA from testis tissue by spin column

Fig.16. RNA from sperm sample by spin column

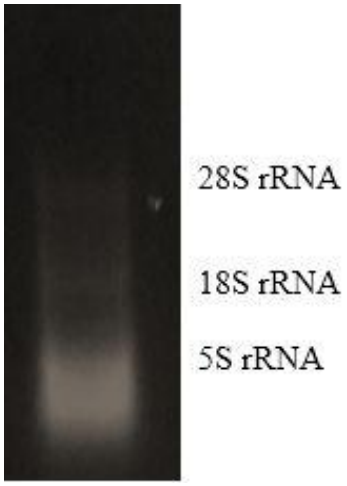

Fig. 17. RNA from sperm sample by hot RNA Xpress reagent method.

RNA isolated by different methods from sperm and tissue were checked for its integrity by resolving on 0.8% Agarose gel and observing in UV transilluminator (Fig. 11-17). Though RNA was isolated from both sperm and tissue by four different conventional methods, one modified method was also used to isolate RNA from both the sample to optimize the isolation procedure especially for Sperm sample, for optimum yield of spermatozoal RNA. In case of modified method, Sperm and tissue sample were heated at 65˚C with RNA-Xpress™ reagent and

processed for homogenization. Periodic vortexing were provided to perform complete dissociation of cell clumps. One additional Chloroform and isoamyl alcohol washing step was used in this case to separate out the proteins and other phenol contaminations that might have retained after first washing with chloroform. As compare to other four methods modified hot RNA-Xpress™ reagent method has shown high quality RNA bands. 28S rRNA, 18S rRNA and 5S RNA bands were clear in case of RNA-Xpress™ reagent. Hot SDS lysis method yielded high amount of RNA which was represented in Agarose gel electrophoresis, but had a significant gDNA contamination, observed near the well. Some amount of DNA contamination was also observed in case of Cold RNA-Xpress™ reagent method. A single band was observed in case of spin column method for both the sperm and tissue sample, which is expected to be 28S rRNA. The presence of single 28S rRNA band in case of spin column method signifies improper lysis of cells which results in small amount of RNA. In most of the methods 18S and 5S bands were sharper than 28s which might be the result of degradation of RNA by RNases from different sources like reagents and tips etc. Prominent 28S rRNA band in case of spin column justifies the purity of RNA, but due to improper homogenization of the sample 18S and 5S rRNA was not visible. Though Hot RNA Xpress reagent method was modified to be an optimised method for Sperm RNA isolation for optimum yield and purity. A260/A280 ratio of 1.9 and 2.1 for sperm and tissue sample meant the RNA was free from protein contamination.

Concentration of RNA isolated from Tissue and sperm sample by different methods

Yield of RNA from testicular tissue and sperm were found to be heighest in hot trizol method, which was 139.5 ng/µL and 38.60 ng/µL respectively. The purity of the extracted RNA by hot trizol method was measured by the A260/A280 ratio, which for testis tissue and sperm were 2.1 and 1.923 respectively. The yield and purity of the extracted RNA by hot trizol method was found to be superior to other extraction methods. The GITC method gave least yield and purity, which. The yield was 56.36 ng/µL and 0.84 ng/µL for tissue and sperm sample respectively. It also showed least purity which was 1.83 and 1.4 respectively. The spin column method yielded 95.12 ng/µL and 3.76 ng/µL respectively. The low yield of RNA from sperm sample can be due to incomplete homogenization of the sperm cells by the lysis buffer as sperm plasma membrane is much more rigid than a somatic cell.

Table 3: Yield of RNA isolated from Tissue and sperm by different methods.

	Tissue					Sperm				
	Hot trizol	Cold trizol	Spin	SDS Lysis	GITC	Hot trizol	Cold trizol	Spin	SDS	GITC
A260	3.488	3.140	2.378	1.969	1.409	0.965	0.618	0.09	0.029	0.021
A280	1.660	1.562	1.495	1.020	0.769	0.501	0.370	0.06	0.015	0.015
A260/ A280	2.1	2.01	1.59	1.930	1.830	1.923	1.67	1.44	1.933	1.40
Yield (ng/µL)	139.5	125.6	95.12	78.76	56.36	38.60	24.72	3.76	1.16	0.840

cDNA Synthesis of RNA isolated from tissue and sperm sample

RNA concentration of sperm was found less as compared to tissue RNA, but after preparation of cDNA no smear was observed in case of Sperm cDNA due to absence of proper RNA template for amplification. This might have revealed the low integrity of RNA sample isolated from sperm which got degraded before preparation of cDNA by RNase activity from different source. GAPDH amplification was done for both the tissue ans sperm cDNA which resulted only amplification of tissue cDNA sample. Sperm cDNA sample was not amplified with GAPDH due to insufficient amount of RNA which was used as template for cDNA. Amplified bands were observed for tissue sample which was approximately 500 bp in length.

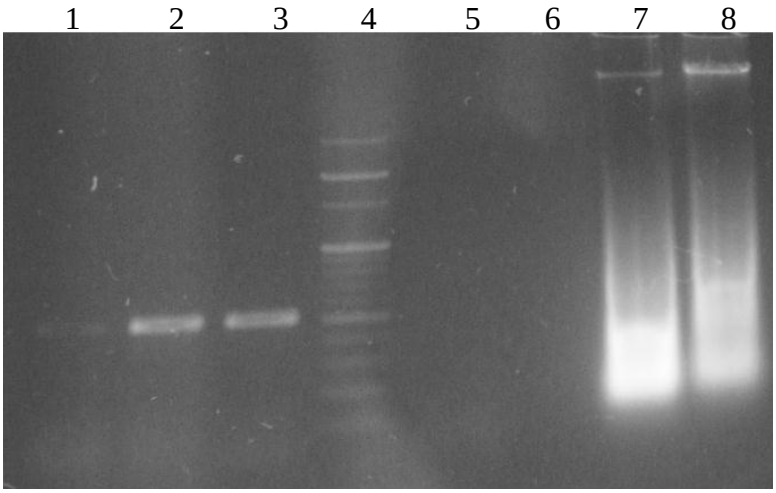

Fig. 18. Synthesis of cDNA and amplification of GAPDH from sperm and tissue sample. 1. Amplified GAPDH from sperm cDNA, 2 &3. Amplified GAPDH from Tissue cDNA 4. 1Kb DNA ladder, 5 & 6. cDNA from Sperm RNA, 7& 8. cDNA from tissue RNA.

Comparison of different RNA isolation methods from sperm

The modified hot trizol method by Hot RNA Xpress Reagent gave best yield and purity for tissue and sperm sample, which was 38.60 ng/µL and 1.923 respectively. The number of steps was found be least in SDS lysis method and highest in spin column method. However time required was found to be least in spin column which was 30 minutes with an overnight incubation and for, SDS lysis method the time was three hours with an overnight incubation. The cost of a preparation was highest in spin column method which was Rs. 250 as it is a commercially available and for SDS lysis method was Rs. 70. The cost for one preparation for hot trizol method was Rs. 100. The yield and purity the hot trizol method with a low cost makes it superior to other methods for extracting RNA from sperm.

Table 4: Different isolation methods for sperm RNA.

Characteristic	Hot SDS lysis	Isolation with RNA Xpress Reagent	GITC method	Spin column method	Modified method (Hot RNA Xpress Reagent)
Lysis Condition	100°C for 5 minutes	Room temperature for 5 minutes	37°C for 5 minutes	Room temperature for 2 minutes	65° C for 15 minutes
Lysis solution	2% SDS 16mM EDTA	RNA Xpress Reagent (Himedia)	Solution D	RNA lysis solution (HRL)	RNA Xpress Reagent
Centrifugation steps, n	4	5	5	6	5
Time	3 hours with overnigh t incubatio n	2 hours with overnight incubation	5 hrs with overnight incubation	30 minutes with overnight incubation	2 hours 40 minutes with overnight incubation
Yield (per $2x10^8$ cells) (ng/µL)	1.16	24.72	0.840	3.76	38.60
Quality (260/280 ratio)	1.923	1.67	1.40	1.446	1.923
Cost (Rs.)	70	100	85	250	100
Availability	Reagents for lysis solution easily available	Commercially available	Reagents for lysis solution easily available	Commercially available	Commercially available
Ease of access	Easy, but poor yield	Easy with good yield	Time consuming with poor yield	Very easy	Easy with good yield

Comparison of different RNA isolation methods from Testis Tissue sample

The modified hot trizol method by Hot RNA Xpress Reagent gave best yield and purity for tissue and sperm sample, which was 139.5 ng/µL and 78.76 respectively. The number of steps was found be least in SDS lysis method and highest in spin column method. However time required was found to be least in spin column which was 30 minutes with an overnight incubation and for, SDS lysis method the time was three hours with an overnight incubation. The cost of a preparation was highest in spin column method which was Rs. 250 as it is a commercially available and for SDS lysis method was Rs. 70. The cost for one preparation for hot trizol method was Rs. 100. The yield and purity of the with a less cost by hot trizol method makes it superior to other methods for extracting RNA from testicular tissue.

Table 5: Different isolation methods for testicular tissue RNA.

Characteristic	**Hot SDS/Phenol**	**Isolation with RNA Xpress Reagent**	**GITC method**	**Spin column method**	**Modified method (Hot RNA Xpress Reagent)**
Lysis Condition	100°C for 5 minutes	Room temperature for 5 minutes	37°C for 5 minutes	Room temperature for 2 minutes	65^{o} C for 15 minutes
Lysis solution	2% SDS 16mM EDTA	RNA Xpress Reagent (Himedia)	Solution D	RNA lysis solution (HRL)	RNA Xpress Reagent (Himedia)
Centrifugation steps, n	4	5	5	6	5
Time	3 hours with overnight incubation	2 hours with overnight incubation	5 hrs with overnight incubation	30 minutes with overnight incubation	2 hours 40 minutes with overnight incubation
Yield (per 50mg of tissue) (ng/µL)	78.76	125.6	56.36	95.12	139.5

Quality (260/280 ratio)	1.930	2.01	1.830	1.59	2.1
Cost (Rs.)	70	100	85	250	100
Availability	Reagents for lysis solution easily available	Commercially available	Reagents for lysis solution easily available	Commerciall y available	Commerciall y available
Ease of access	Easy, but poor yield	Easy with good yield	Time consuming with poor yield	Very easy	Easy with good yield

Chapter 6

Discussion

RNA was isolated from goat testicular tissue sperm. Isolation was carried out by five different methods, namely, Guanidium Isothiocyanate method, SDS Lysis Method, cold Trizol Method, Spin column method and a modified hot Trizol method. Yield and protein contamination of all the methods for testicular tissue and sperm were compare. Five methods were compared on the basis of yield, time, cost, availability, lysis condition and ease of access for testicular tissue and sperm. From table 5, it was observed RNA isolated by Hot RNA Xpress reagent and Cold RNA Xpress reagent yielded maximum RNA, to other four methods. A260/A280 ratio more than 1.8 meant "pure" RNA, free from protein contamination. Comparison of yield and purity of the isolated RNA by all the five methods, clearly showed the superiority of the Hot Trizol method. Though spin column method is an optimized procedure for RNA isolation still it yielded low RNA, the reason for which is incomplete lysis of the samples. Besides sperm plasma membrane is highly rigid and the lysis solution (HRL) supplied with the kit was unable to disrupt the plasma membrane properly, and the sperm cells clogged the spin column.

The study revealed that the hot trizol method of heating Trizol at 65^{o} C for 15 minutes and an optimum 200 million sperm cells was optimum for maximum recovery of RNA from goat spermatozoa. Electrophoretic and spectral analysis revealed high quality RNA. Higher yield due to improved sperm cell lysis was achieved with hot trizol. Hot RNA-Xpress™ reagent was able to lyase the rigid plasma membrane of sperm cells. An additional chloroform separation step provided maximum protein denaturation.

Total RNA was subjected to reverse transcription and amplification with GAPDH primer. Tissue mRNA was amplified and its size was found to be 500 bp. Sperm mRNA amplification showed a light band, which confirms the presence of low mRNA in total RNA obtained from sperm samples.

Chapter 7

Conclusion

Isolation of good quality sperm RNA can be used as an important tool for analyzing fertility related issues. Spermatozoa are known to contain both coding RNAs and non-coding RNAs such as miRNAs. Knowing the identity, functions and regulation of these RNA will set up a ground work upon which missing spermatozoal RNA may be identified as a factor for idiopathic infertility. It will also provide fundamental knowledge for developing newer ARTs, studying reproductive diseases, developing RNA-based contraceptives, and constructing spermatozoal expressed sequence tags. The study was aimed to compare different RNA isolation methods from sperm and testicular tissue. A suitable sperm purification technique is required as sperm RNA yield is strongly correlated with the number of sperms. Goat (*Capra hircus*) semen was purified by swim-up method. Motile sperm were selected for the process of RNA isolation. Four different RNA isolation methods namely cold Trizol, Gunidinium thiocyanate method and SDS lysis method and silica column based spin column method were used to isolate total RNA from sperm and testicular tissue. The hot Trizol method yielded good amount of RNA with less contamination of DNA and proteins. The modification of heating the sample in Trizol and an additional chloroform phase separation step increased the yield of total RNA. The modified method yielded highest amount of RNA for both tissue and sperm samples which was 139.5 ng/µL and 38.6 ng/µL respectively. The RNA integrity was high as was defined by a sharp 28S and 18S rRNA band in the agarose gel. The A260/A280 ratio was 2.1 and 1.923 for tissue and sperm samples which signifies purity of RNA. The mRNA was also reverse transcribed and GAPDH amplified. A modified Trizol method was developed which resulted in increased yield (38.60 ng/µL) and purity (A260/A280 : 1.923).

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Appendix

I. Preparation of reagents

1. **0.9% (wt./v) saline:** 0.9gm Sodium Chloride A.R. (Hi-Media, Mumbai, GRM853) was dissolved in 100 ml of distilled water and stored at room temperature.
2. **70% (v/v) Ethanol**: 70 ml Molecular biology (MB) grade ethanol (Hi-Media, Mumbai, MB106) was diluted with 30 ml distilled water and stored at 4˚C.
3. **0.1 % DEPC water:** 1 ml DEPC (Hi-Media, Mumbai, MB076) is added to 1000 ml distilled water, incubated overnight at 37˚C and autoclaved. Stored at room temperature.
4. **0.8 % Agarose Gel:** 0.8gm of Molecular biology grade Agarose low EEO (Hi-Media, Mumbai, MB002) was dissolved in 100 ml of 1X TAE buffer with 2 µl of Ethidium Bromide (Hi-Media, Mumbai, MB074).
5. **50 X TAE buffer:**
   24.2 g Tris free base (Hi-Media, Mumbai, MB029), 5.71 ml Glacial Acetic acid L.R. (Hi-Media, Mumbai, AS119), 10 ml of 0.5 M EDTA (Amresco, Dallas, E117) was mixed in 80ml of distilled water and volume was made up to 100 ml with DEPC treated water. This was further diluted to 1X concentration before use.
6. **Solution A**: 4 M GITC (Hi-Media, Mumbai, MB015); 25mM sodium citrate (Hi-Media, Mumbai, TC249); 0.5% sarcosyl (Hi-Media, Mumbai, MB108) was prepared and volume made up to 10ml.
7. **Solution B**: 0.72µl of β-mercaptoethanol (Hi-Media, Mumbai, MB041) was added to 10 ml of solution B.
8. **2 M sodium acetate preparation:** 6.6ml 3M sodium acetate was added with 3.4ml nuclease-free-DEPC treated water (Hi-Media, Mumbai, TCL016) and stored at room temperature.
9. **SDS lysis solution:** 2% SDS (1ml of 20% SDS (Hi-Media, Mumbai, ML007)) + 16mM EDTA (320 µl of 0.5 M EDTA (Amresco, Dallas, E117)) was mixed and volume was made to 10 ml using nuclease free DEPC treated water (Hi-Media, Mumbai, TCL016).
10. **Preparation of reagents for spin-column methods:** 10µl 2-mercaptoethanol (Hi-Media, Mumbai, MB041) was added with 1ml of Lysis solution .Wash solution II dilution: 1ml WS solution was diluted with 3ml of 100% Ethanol (Hi-Media, Mumbai, MB106).

II. Equipments

I.	Digital Weighing Balance	(Wensar, PGB200)
II.	MultiskanGO (Spectrophotometer)	(Thermo Fisher Scientific, 51119200)
III.	Ultracentrifuge 2-16KL	(Sigma, 146109)
IV.	Thermocycler (96 Well) (Veriti)	(Applied Biosystems)
V.	Horizontal Electrophoresis Unit	(Genei, F0133)
VI.	High Performance UV Transilluminator	(UVP, P/N 95-0423-20)
VII.	G-Fuge	(Genetix, GX-8001RC)
VIII.	Mini Centrifuge	(REMI, C852)
IX.	Heating Dry Bath	(Genetix, GX-3215810)
X.	Inverted microscope.	(Primo vert, Zeiss)